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14. ABSTRACT

Over the past year, we have optimized various systems for the identification of tumor-reactive T cells, and generated potentially tumor-reactive T cell clones from a number of patient samples. We have also optimized protocols for antigen identification and TCR repertoire analysis. We are hopeful that we are close to verifying there are several clones generated in recently screened patients (such as BC81 mentioned in this report). We are now beginning to optimize assays to test T cell killing of target cells. These include LDH release based cytotoxicity assay and live cell imaging. Once we have identified tumor reactive T cells, we will then examine their levels in patient tissue by the use of histology, TCR repertoire analysis, and archived tissue samples.

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Annual Progress Report 10/16/2013 – 10/15/2014
DoD Multi-Team Award
Enhancing the Breadth and Efficacy of Therapeutic Vaccines for Breast Cancer
Peter P. Lee, M.D.
City of Hope Cancer Center, Duarte, CA

INTRODUCTION

The immune response offers exquisite specificity and the potential to target tumor cells without harming normal cells. Inducing an effective immune response via therapeutic vaccines for cancer had been a promising but elusive goal for years. For breast cancer (BC), vaccine efforts have largely focused on eliciting immune responses to HER2. While HER2 is generally assumed to be a good antigen in HER2-overexpressing tumors, HER2-specific T cells exist at very low levels (less than 0.1%) in peripheral blood of such patients (Inokuma, dela Rosa et al. 2007). Hierarchy of the T cell repertoire and negative selection can shape immune responses in ways not readily predictable from protein expression levels alone. Thus, targeting a single antigen such as HER2 in breast cancer is likely to be insufficient - instead we need a repertoire of multiple immunologically validated T cell antigens present in breast cancers that can be deployed in a patient-specific manner. Research has focused on stimulating T cells using many pathways including the T cell antigen receptor (TCR), via co-stimulatory pathways, and manipulating the tumor environment. To optimally activate pre-existing anti-tumor T cells in BC patients, the antigens to which these T cells target must be determined. It is now recognized that invasive ductal carcinoma of the breast is a heterogeneous disease consisting of several major molecularly defined subtypes, including Luminal A, Luminal B, HER2+, and Basal (also known as 'triple-negative', and includes the 'claudin-low' subset). These subtypes have distinct clinical, genomic and proteomic features, and it is becoming clear that there are differences between BC subtype and response to specific therapeutic agent. These results, combined with the differences in gene expression that define the distinct subtypes, make it likely that each BC subtype elicits immune responses via distinct sets of antigens, and may evade T cell-mediated killing by distinct mechanisms. Based on these newly discovered features of BC and the host immune response, this project seeks to develop a robust portfolio of immunologically validated antigens for the major BC subtypes, including those that target breast cancer stem cells, that can be used in a patient-specific manner for therapeutic vaccination, as well as to identify drugs that can synergize with these novel immunotherapies. The ultimate goal is to match these antigens and drugs to each patient's tumor subtype, thereby treating each patient with the most potent combinations and opening the door to personalized immunotherapy for breast cancer. This multi-ream project will use a number of novel immunological approaches to look for evidence of BC subtype specific tumor-reactive T cells within the tumor and/or tumor-draining lymph nodes (TDLNs) including isolating, expanding and cloning tumor-reactive T cells which will culminate in a robust portfolio of immunologically validated antigens for the major breast cancer subtypes, including those that target breast cancer stem cells. We seek to expand and enhance the function of these pre-existing anti-tumor T cells in patients by discovering their natural antigens, and identifying mimotopes that broadly activate them with even higher potency. Furthermore, we will enhance the efficacy of these T cells by identifying existing drugs that promote cancer cell apoptosis but have little or no negative effect on T cells. All of these antigens and agents can be matched to each patient's tumor subtype and other molecular characteristics, thereby opening the door to personalized immunotherapy.

BODY:

Our team at City of Hope (CoH) consists of 1 assistant research professor, 1 post-doctoral fellow, and 3 research associates. We work closely with our surgery, medical oncology, and pathology colleagues via an honest broker to obtain samples from the operating room to pathology and to my laboratory. In addition, we continually refine our protocols to maximize recovery of immune cells from tumor and lymph node specimens, and to optimize methods for analysis of fresh samples by flow cytometry. Below is a summary of our progress in relation to our proposed SOW tasks:

Identify immunologically validated antigens by determining antigens recognized by anti-tumor T cells from patients with major subtypes of breast cancer.

1. Generate reagents and identify conditions for experiments to follow: months 1-40, Lee, Slansky, and Spellman
2. Enroll 100 patients with all major breast cancer subtypes from the City of Hope Cancer Center (CoH): months 1-36, Lee
3. Process patient samples (blood, TDLNs, tumor): months 1-38, Lee
4. Identify and isolate anti-tumor T cells from TDLNs and tumor samples: months 1-40, Lee
5. Generation and initial analysis of T cell clones: months 1-40, Lee
6. Determine antigens as subtype-specific, stem-specific, or shared (Aim 4a): months 12- 40, Lee, Slansky and Spellman
7. Identify antigens that target breast cancer stem cells (Aim 3b): months 12-40, Lee, Slansky and Spellman

Patient Enrollment and Sample Acquisition

Our progress thus far has focused on tasks 1-5. As of January 2014, we have acquired 16 breast cancer patients' specimens consisting of peripheral blood, lymph node and/or tumor. From those 16 patients, we have sent out 94 samples to the Slansky/Kappler group for TCR sequencing. Obtaining blood is essential to this study to generate source cells for both monocyte-derived dendritic cells, which will be used as autologous antigen presenting cells (APCs) to present tumor antigens to T cells. Patient characteristics are summarized in Table 1. All participants were without a history of any immune disorder prior to breast cancer diagnosis and had their surgical treatments at City of Hope (CoH). Through an honest broker, written informed consent had been obtained from all participants according to CoH and HIPAA regulations using a tissue banking protocol. Patient peripheral blood samples, breast tumor tissue, and/or tumor draining lymph node (TDLN: non-sentinel lymph node and/or sentinel lymph node) were collected and have been utilized for research purposes.

The Slansky/Kappler and Lee teams have recently re-designed a flow chart for the distribution of patient T cells between labs (Figures 1). The flow chart represents an ideal situation, which are heavily dependent on the sample sizes we receive and success of upstream protocols.

Currently, our approach to identifying reactive T cells is focused solely on CD8+ T cells. While we are still actively supplying CD4+ T cells for TCR repertoire analysis to the Denver team, we have decided it is more efficient and productive to focus our efforts on CD8+ T cells. This is primarily due to the fact that most of our protocols and tools (antibodies, identified peptides, and peptide libraries) are restricted to HLA-A2, which is only useful for CD8+ T cells. Our original plan to focus on HLA-DR4 restricted CD4s has proven difficult since there is no publically available antibody for HLA-DR4 and since very few of our HLA-A2+ patients have also been HLA-DR4+ (Table2). Thus, for all these reasons we now primarily focus on CD8+ T cells in our patient samples.

Process of Patient Samples

<u>HLA typing</u>

We have arranged to have patients' peripheral blood mononuclear cells (PBMCs) DNA HLA typed through the Histocompatiblity lab here at CoH. CoH's Histocompatibility Laboratory is fully accredited by The American Society of Histocompatibility and Immunogenetics (ASHI), College of American Pathologists (CAP), and Clinical Laboratory Improvement Amendments (CLIA 88). They will carry out the typing using the sequence-specific oligonucleotide probe (SSOP) method. The SSOP method allows the HLA lab to define the HLA type of our patient subjects to the allele level (so called '4 digits'). Initially we are requesting for the allele level

typing of subjects for only HLA-A2 and HLA-DR, but information on other alleles is available at a later date if desired. Since the beginning of the year, we have sent 16 samples to be HLA typed at CoH (Table 2).

Identify and isolate anti-tumor T cells from TDLNs and tumor samples

Cell Isolation

We are currently using the Stem cell Technologies bead based isolation kits for positive selection of T cells (CD8+ and CD4+) and monocytes (CD14+). Once patient samples are prepared into single cell suspensions, we plate them and leave them overnight on a matrigel. The matrigel helps clean up the population by getting rid of debris as well as allowing tumor cells to adhere to it. After an overnight incubation, we proceed with CD8+ isolations followed by CD4+ isolations for both tumor and LN samples (Figure 2). In order to obtain sufficient numbers of T cell from TDLN and tumor specimens, we isolate T cells from the entire sample. However since we receive significantly more tissue from blood, we only isolate T cells from approximately one third of the total number of PBMC's we collect. From PBMC's, we isolate CD14+ cells first, followed by CD8+ and CD4+. Cell numbers vary depending on patients; therefore not all experiments can be performed due to low cell numbers. In order to acquire the most information possible, we have made a priority list. The priority list demonstrates the number of cells needed for each of the experiments listed in order from highest to lowest priority (Table 3). Once T cells are isolated, they are cultured with IL-2 (50IU/ml) and are not expanded due to possible skewing of the T cell population. T cells are fed 50IU/ml of IL-2 every other day until they are screened for tumor reactivity. After T cell isolations, purity is examined using Flow Cytometry.

Generation of Antigen Presenting Cells (APCs)

The generation of antigen presenting cells (APCs) is a critical component of this project. As mentioned previously, we identified two possible sources of APCs: Epstein Barr Virus (EBV) transformed B cells and monocyte-derived dendritic cells (mDCs), both of which can be generated from patient autologous PBMCs. DCs are potent professional APCs. We have found that we have been able to generate a more than sufficient number of DCs necessary for the low number of TDLN and tumor T cells we are working with. Thus, we are able to cryopreserve a significant amount of patient PBMCs for later use as a monocyte-derived dendritic source. This coupled with difficulty we have had in reproducibly generating EBV transformed B cells and the fact that B cells do not naturally phagocytose antigen led us to focus solely on using mDCs as an antigen source.

To generate mDCs, isolated monocytes are cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (O'Neill and Bhardwaj 2005). After 5-6 days of culture, immature DCs are matured overnight using a monocyte maturation cocktail (MMC) composed of 5ng/ml IL-1β, 150ng/ml IL-6, 5ng/ml TNF-α and 1ug/ml prostaglandin E2 (PGE2). Once mDCs are generated they will be used as APCs for tumor antigen to patient T cells.

T cell sorting and clone culture conditions

To improve our cloning efficiency, we have slightly altered our T cell sorting and cloning conditions. First, we have increased the serum concentration from the media we sort from to 30% FBS versus 2% FBS as before, providing more nourishment prior to undergoing the harsh sorting process. Second, we are using a larger sort nozzle (100um) than before (70um). This reduces the pressure on the T cells as they go through the sorting process. Third, based on optimization experiments using healthy donor PBMCs where we compared media conditions consisting of IMDM with a) 10% Human AB serum (HS), b) heat-inactivated 10% Human AB serum (HIHS), c) 10% Fetal Bovine serum (FBS) + 2% HS and, d) heat-inactivated 10% FBS (HIFBS) + 2% heat-inactivated HS (HIHS) (Figure 3), we have altered the concentration of serum in our T cell media from containing 10% human AB serum (HS) to 10% FBS + 2% HS. Fourth, we have added additional supplements to our T cell media, i.e., sodium pyruvate, non-essential amino acids, and β-mercaptoethanol, to provide additional energy and redox agents for T cells to metabolize during the proliferation/cloning process. Last, after

single cell sorting T cells into a 96 well plate, we do a 'flash spin' to bring down any droplets which may be adhering to the side of the well into the culture media to ensure that the T cell is in contact with the media within the well.

By modifying the T cell clone culturing conditions, our cloning efficiency is roughly similar as before. In our experience, while optimizing different T cell cloning conditions on healthy PBMCs, we have observed a donor-to-donor variation in T cell cloning responses (Figure 3), therefore we do expect donor-to-donor cloning efficiencies to vary.

Sequencing of TCR of tumor reactive T cell clones

Sequencing of T cell clones

Because healthy donors' PBMC feeder cells contain T cells, it is imperative that we are able to distinguish between the breast cancer patient's CD8 T cells from the donor's CD8 feeder cells for TCR sequencing. Since we are identifying HLA-A2 positive breast cancer patients to find tumor-reactive T cells, we can screen healthy donors' PBMCs for HLA-A2 positivity via FACS and identify which healthy donors are HLA-A2 negative. We can then stain the T cell clones with HLA-A2 and CD8 antibodies to differentiate the feeder cells from the growing clones (Figure 4) using a high-through put method of flow cytometry. Taking x-ul of the T cell clones, staining with HLA-A2 and CD8 antibodies, and acquiring a x- volume using a 96-well plate on the Fortessa flow cytometer, we can back calculate and determine the total number of HLA-A2+ CD8+ T cell clones. We can then identify the cell numbers needed to, a) re-screen and confirm tumor reactivity, b) re-expand and cryopreserve, and/or c) decide whether to terminate that clone.

At COH, we are sequencing the TCR receptor of potential tumor antigen reactive clones for each cancer patient. This information will guide us to generate full length recombinant TCR proteins that will be used to screen against the bacteriophage peptide library the Slansky/Kappler team has created to identify unknown antigens. Also, the alpha-beta TCR sequence from autologous tumor-reactive T cell clones will be cross referenced with the T cell repertoire data provided by Slansky/Kappler team, to evaluate the robustness of the high throughput T cell repertoire approach in identifying TCR sequence of tumor reactive T cells. In order to simultaneously sequence alpha and beta genes of tumor reactive T cell clones, we amplified and fused Vα and Vβ amplicons during the PCR reaction and enriched the paired product during second PCR (Turchaninova, MA, *et al*., 2013). Briefly, RNA is extracted from tumor reactive clones and converted into cDNA using oligo(dT) followed by amplification TCR alpha and beta genes using multiplex primer mixture for priming Vα ,Vβ, Cα and Cβ (provided by the Slansky/Kappler team) . Vα and Vβ primers have an overlapping sequence that facilitates fusion of alpha and beta TCR genes. A small aliquot from the first PCR reaction was used to perform the second PCR reaction using nested primers T3-α and T7-β that spans the constant region of Cα and Cβ. The amplified product (Figure 5) can be visualized by agarose gel electrophoresis. The expected band is then excised and purified using a gel extraction kit and submitted for Sanger sequencing using T3 or T7 primers to the CoH Integrative genomics core. The TCR nucleotide sequences were analyzed using IMGT/V-QUEST to determine the identity of the V alpha and V beta of the TCR.

Growth of Autologous Cancer Cells

Autologous cancer cells grown *in-vitro* are an ideal target source for identification of bonafide cancer reactive T cell clones. We are currently optimizing methods to perpetuate autologous cancer cells. Our tumor cell culture procedures involve cutting tissues into small pieces and mincing, followed by digestion. We typically use 5 ml of media to digest and add Liberase from Roche and Dnase I from sigma and digest in the incubator for 45min-60 min. Constant rotation of the tissue is more effective for digestion and obtaining a single cell suspension. The tissue is then filtered though a 100um filter followed by a 70um filter and the single cell suspension is resuspended in tumor growth media. The tumors cells are seeded on matrigel coated plates in DMEMF12 advanced media containing 5% fetal bovine serum, 0.4 μg/mL hydrocortisone, 5 μg/mL insulin, 10 ng/mL EGF,

100ng/ml cholera toxin, 1% pen/strep with rock inhibitor (5μmol/L) (Liu Xuefeng, *et al.,* 2012). Further expansion of tumor cells were done using a trans well approach (Figure 6), where the tumor cells were placed on the bottom of the plate and human irradiated fibroblast feeder cells are inserted into the trans well itself, which provide factors that support tumor growth (Figure 7). Occasionally when stromal cell growth is observed, differential tyrpsinization is done to separate stroma from tumor cells in order to enrich the cancer cell population of epithelial origin.

Generation and analysis of T cell clones

Generation of Tumor Reactive T cells

Over the last year we have optimized conditions for the identification of tumor reactive T cells. Our two primary strategies are to use mDCs pulsed with breast cancer cell line lysate and autologous tumor cells as targets. In the previous year's progress report we discussed the possibility of using electroporation of either cell line lysate or RNA into mDCs for use as a target. However, we found that the number of cells necessary to generate the amount of RNA needed was unrealistic. Furthermore we were able to identify conditions using extracellular lysate as antigen and allowed us to discontinue our electroporation attempts.

Using a CMV specific T cell clone previously generated in our lab (clone 499C.35), we identified that mDCs grown for 5-7 days in culture, pulsed overnight with 200ug/ml of a cell lysate made from CMV infected cells (lysate purchased from Microbix), and then matured overnight with a MMC cocktail could effectively elicit a CD107 mobilization response in 20% of the CD8 T cell clone pool (Figure 8). While this is less than the 71% that peptide pulsed mDCs elicit, we do not expect a cell lysate that is extremely diluted with a multitude of other proteins and peptides to produce the same response in reactive T cells as concentrated cognate peptide. We then went on to show that CMV lysate pulsed mDCs could be used to identify CMV reactive T cells in healthy donors in both CD8 (Figure 9) and CD4 (Figure 10) T cells as show by CD137 staining after a 24 hour co-culture of mDCs and T cells. Note that in this case the lysate pulsed mDCs elicited a higher T cell response than the pp65 peptide pulsed mDCs since the lysate pulsed mDCs are capable of identifying a polyclonal T cell response to many CMV peptides. As a result of this work, we now use 5-7 day generated autologous patient mDCs cultured overnight with 200ug/ml of breast cancer line lysate (prepared by the Spellman group) and then matured overnight with MMC to screen for T cells reactive to breast cancer antigens.

In parallel to using the mDC approach, we have been routinely using cultured autologous tumor cells as a target for identification of tumor reactive T cells. Tumor cells cultured for 5-7 days, as described above, are then treated overnight with 1,000 IU/ml of IFNy to upregulate MHC Class I expression, which is known to be down-regulated on tumor cells (Figure 11A). In addition, as described in the literature, this treatment upregulates PD-L1 expression (Figure 11B). Because of this we have also incorporated PD-1 and PD-L1 blocking antibodies into our autologous tumor – T cell co-cultures in case PD-1 ligation inhibits the ability of T cells to react against tumor targets. So far, however, it appears that PD-1 blocking in breast cancer patient samples does not significantly enhance reactivity to tumor cells (Figure 12)

To identify tumor reactive T cells we currently use CD137 (4-1BB) as an activation marker for sorting. This molecule is upregulated between 15 and 24 hours after a T cell has encountered its cognate antigen. Compared to CD107 mobilization, which peaks at 4-6 hours, we have found CD137 background levels to be lower in unstimulated T cells, which is important for identification of reactive T cells in a polyclonal setting where reactive cell numbers are expected to be low. However, we are currently optimizing a staining panel to include an IFN-γ capture assay and live cell TNF-α secretion as further markers of activation. We hope this panel will help us identify truly reactive T cells and maximize the downstream productivity in re-screening sorted T cells. Patient BC81 demonstrates an ideal reactivity experiment and a common sorting setup, with unstimulated T cells and unpulsed mDCs as negative controls and CD3/CD28 stimulated T cells as positive controls (Figure 12). T cells identified as CD137+ upon stimulation with either mDCs pulsed with BC cell line lysate or autologous tumor cells are sorted for expansion and then later re-screened against the same target. Recently, we

have switched from single cell sorting of reactive T cells to bulk sorting of reactive T cells into the same well. We found that we were spending significant amounts of time maintaining hundreds of T cell clones from many patients, many of which tested negative upon a reconfirmation screening to target tumor cells. Switching to bulk sorting and culturing simply enables us to be more efficient with our time and resources.

To date we have reacted PBMC, TDLN, and/or tumor T cells against either mDC or tumor targets from 9 patient samples. Currently we are in the middle of rescreening sorted T cells against their targets for confirmation of their reactivity. Summary of the results of initial reactivity sorts from PBMC and TDLN T cells are shown (Figure 13). So far it appears that the number of reactive T cells in BC patients is low (less than 5%). We suspect this may largely be due to the well-described dysfunction and anergy of tumor specific T cells in patients. We are however optimistic that continued screening of patient samples with our established methods will yield a few patient samples, such as BC81, with high levels of reactive T cells.

Expansion of T cells for Putative and Predicted Antigen Screening

In our previous progress report we discussed screening patient T cells against artificial APCs transfected with putative BC antigens that the Denver team has prepared. Due to the low number of T cells we acquire from patient TDLN and tumor samples, we have found this difficult to do in parallel with our other reactivity assays. Instead, we are now taking aliquots of patient T cells and expanding them to a high number. These T cells can then be used for screening against those putative targets as well as used by the Portland team for screening against the predicted antigens they have identified from BC cell lines.

Direct T cell Cloning

In our last report we discussed potentially identifying reactive T cell clones by directly single cell cloning T cells from patient tumor tissue. To generate dominant T cell clones in culture this would statistically require production of 200-300 clones. We pursued this strategy on several patients but found it to be unproductive. T cell cloning efficiency of tumor T cells in our hands is around 5% of T cells sorted. Thus generation of 300 clones requires single cell sorting of 6,000 clones. This proves to be infeasible in terms of the workload for maintaining those sorted clones and the amount of T cells per sample it would require. Furthermore the sequencing of each individual T cell clone (in order to determine which T cell clones have a unique TCR sequence) is laborious and time consuming and unrealistic for so many T cells. Perhaps most importantly it became clear that that there is no guarantee that a given T cell from a patient will be HLA-A2 restricted, as it could be restricted to one of the patients other 5 HLA class I molecules. Without this information it would therefore be impossible to use the Denver team bacteriophage peptide library for HLA-A2 to screen these T cells. As a result we have abandoned this approach in order to focus our time more efficiently in other areas of the project.

Summary of Plans for Future Work

Project team members have recently returned from a productive meeting with our collaborators at OHSU. The trip allowed for excellent extended discussion of experimental approaches and strategies.

As such, we have outlined an action list for each team to carry out over the next six-12 months. The primary focus of the CoH team is to continue our search for tumor reactive T cells in BC patients. We are hopeful that we are close to verifying there are several clones generated in some recently screened patients (such as BC81 mentioned above). We are now beginning to optimize assays to test T cell killing of target cells. These include LDH release based cytotoxicity assay and live cell imaging. Once we have identified tumor reactive T cells, we will then examine their levels in patient tissue by the use of IHC, TCR repertoire analysis, and archived tissue samples.

Outline of the project plan for the next 12 months

- Continue to screen patient T cells for reactivity to autologous tumor and BC cell line lysate
- Continue to send patient T cells to Denver for TCR repertoire analysis
- Generate T cell clones via FACS sorting that are reactive to tumor antigen
- Optimize cell numbers and methods for confirmation of T cell killing of target cells
- Sequence TCR CDR3 region of identified tumor reactive T cells

Personnel

1. Peter P. Lee, MD – project PI (40% effort)
2. John Yim, MD – CoH Surgical Oncology (5% effort)
3. Joanne Mortimer, MD – CoH Medical Oncology (no salary requested)
4. Jing Zhai, MD, PhD – CoH Pathology (no salary requested)
5. Sailesh Pillai, PhD – Assistant Research Professor (no salary requested)
6. Colt Egelston, PhD – post doc (100% effort)
7. Diana Simons – Research Associate II (95% effort)
8. Grace Jimenez – Lab Technician (100% effort)
9. Emily Andersen – Research Associate I (39% effort to June 2014)
10. Sara Moeller – Research Associate I (14% effort since June 2014)

KEY RESEARCH ACCOMPLISHMENTS

- Optimized protocols for using dendritic cell presentation of cancer cell line lysate antigen
- Optimized conditions for successful culture of autologous tumor cells
- Optimized flow cytometry based identification and sorting of antigen-reactive T cells using CD137
- Optimized improved sorting conditions of low numbers of T cells
- Isolated and sent CD8 and CD4 T cells from 16 patients and 96 samples to Denver for TCR repertoire analysis
- Screened 9 patients for tumor reactive T cells and sorted those T cells
- Begun sequencing the TCRs of generated T cell clones
- Begun screening hundreds of generated T cell clones for confirmation of reactivity

REPORTABLE OUTCOMES

None at this time, but each team is hopeful for the submission of at least 1-2 papers each within the next six months.

CONCLUSION:

Over the last 12 months of this award, we have focused on the identification of tumor-reactive T cells. We have worked out conditions for using both autologous mDCs and autologous tumor cells as targets. Recently we have finalized efficient and optimized methods to carry out these strategies that will enable us to screen many patients in the coming months and therefore to identify and produce several tumor reactive T cell clones soon. The identification of these T cells will lead to downstream antigen discovery.

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APPENDICES:
None at this time

SUPPORTING DATA:

Table 1. Patient characteristics

Code	Prior Therapy	Diagnosis	ER/PR/Her2	Molecular Subtype	Stage
BC54	N	INV. Lobular	+/+/-	Lum A	IIIa
BC55	N	Brain Met	-/-/+	Her2	IV
BC57	N	IDC	+/+/-	Lum A	Iib
BC61	N	IDC	+/+/-	Lum A	IIa
BC67	N	INV. Lobular	+/+/-	Lum A	IIIa
BC70	Y	IDC/BRAIN MET	-/-/-	Basal	IV
BC71	N	IDC/DCIS	-/-/-	Basal	Ia
BC72	Y	Recurrence	-/-/-	Basal	Ia
BC80	N	IDC	+/+/-	Lum A	IIa
BC81	N	IDC	+/+/-	Lum A	Ib
BC85	N	IDC	+/-/-	Lum A	IIa
BC86	N	IDC	+/+/-	Lum A	Ia
BC87	N	IDC	+/+/-	Lum A	IIIa
BC92	N	IDC	+/+/-	Lum A	Ia
BC94	N	ILC	+/+/-	Lum A	IIa
BC96	N	IDC	+/+/-	Lum A	IIa

Table 2: Samples sent for HLA typing since January 2014

Sample Label	HLA-A Type	HLA-DRB1 Type
BC54	02:01	04:01 15:01
BC55	02:01 03:01	12:01 13:01
BC57	01:01 02:01	03:01 07:01
BC61	02:02 30:02	03:01 07:01
BC67	02:01 01:01	3:01, 04:04
BC70	02:01 32:02	04:04 15:01
BC71	02:01	07:01
BC72	02:03 03:01	04:05 13:06
BC80	02:06 33:01	04:07 11:05
BC81	01:01 02:01	04:01 13:01
BC85	02:01 03:01	04:03 11:02
BC86	02:01 31:01	08:01 14:06
BC87	N/A	N/A
BC92	02:06 74:01	04:07 07:01
BC94	02:01 23:01	03:01 04:01
BC96	02:01 03:01	1:01 13:03

N/A: not applicable due to limited material

Table 3. Number of T cells needed for experiments listed from highest to lowest priority (cell numbers are in millions)

Highest Priority → **Lowest Priority**

PBMCs	scSeq	Flow Purity	Tumor Rxn Screen	DC + Lysates Screen	Future Antigen Screening		Total
CD8 T cells	0.1	0.01	0.1	0.1	0.1		0.41
CD4 T cells	0.1	0.01	NA	NA	NA		0.11
CD14 mDCs				0.5			0.50
LN							
CD8 T cells	0.05	0.01	0.1	0.1	0.02		0.28
CD4 T cells	0.05	0.01	NA	NA	NA		0.06
Tumor							
CD8 T cells	0.05	0.01	0.1	0.1	0.02		0.28
CD4 T cells	0.05	0.01	NA	NA	NA		0.06

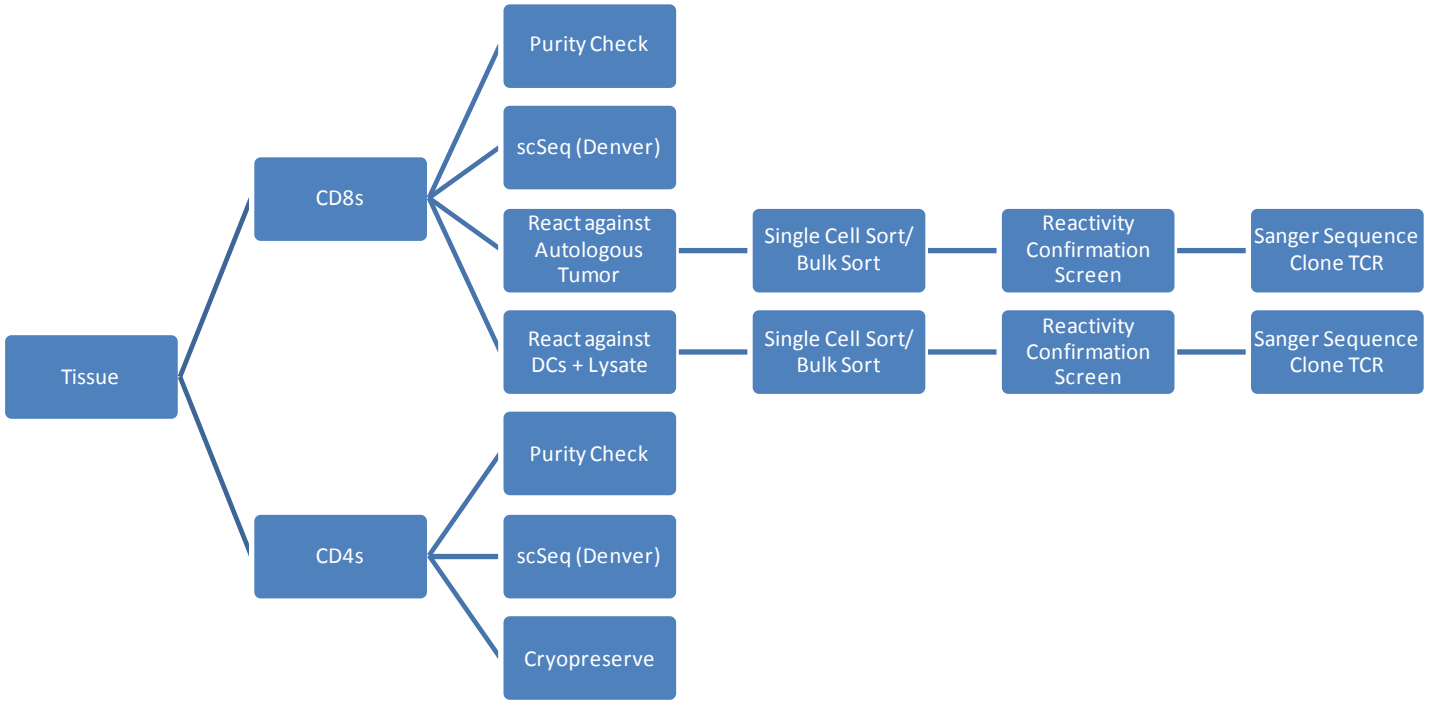

Figure 1. Flow chart demonstrating the distribution of patient T cells between the Slansky/Kappler and Lee labs (scSeq: single cell sequencing; TCR: T cell receptor).

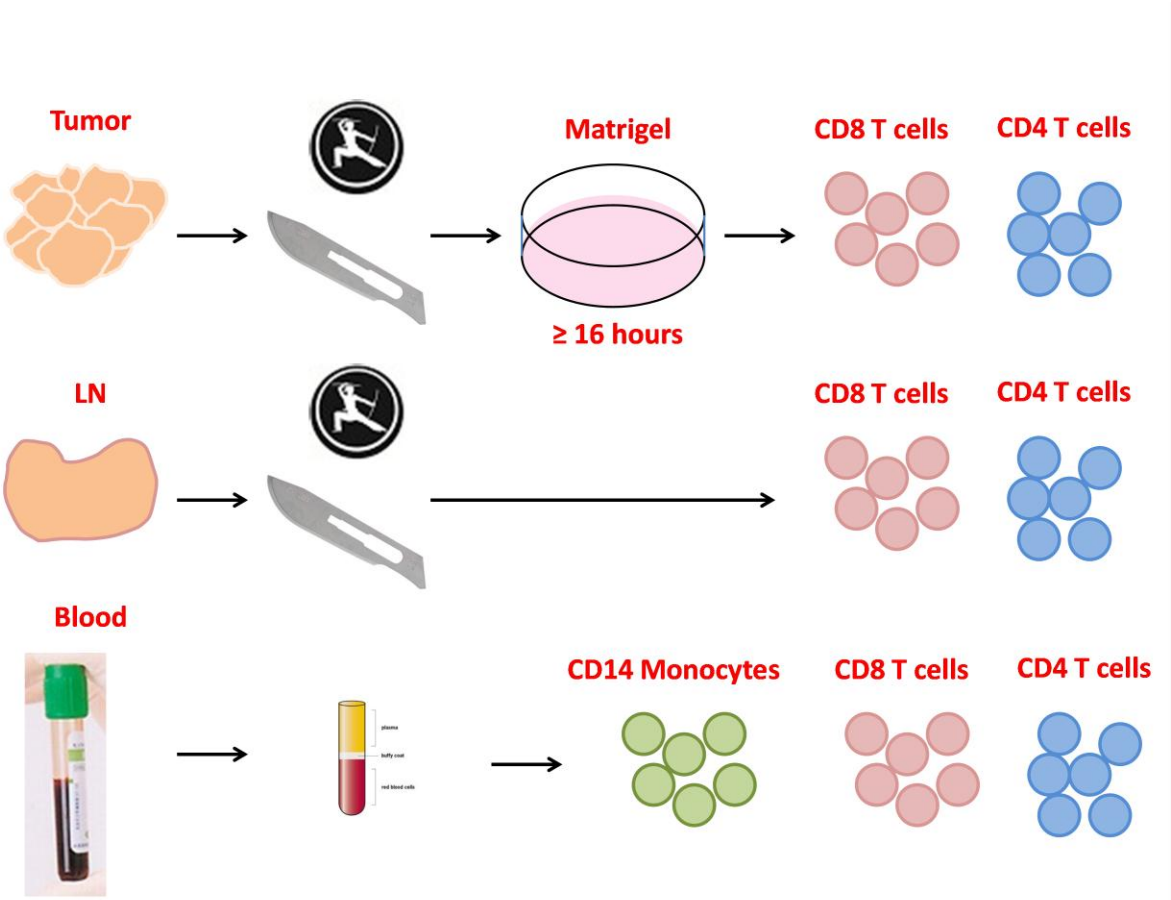

Figure 2. Tissue pipeline for T cell isolations from tumor, lymph node, and peripheral blood.
After single cell suspensions are placed on matrigel overnight, CD14+ monocytes and T cells (both CD8+ and CD4+) are positively selected from blood. Additionally, CD8+ and CD4+ are positively selected from tumor and/or LNs.

A.

	10%HS	10%HIHS	10%FBS 2%HS	10%HIFBS 2%HIHS
Donor A	3	4	1	7
Donor B	4	5	3	19
Donor C	3	3	10	6
	n=10	n=12	n=14	n=32
	5.5%	**6.67%**	**7.78%**	**17.78%**

Total Cloning Efficiency: 9.4%

B.

	10%HS	10%HIHS	10%FBS 2%HS	10%HIFBS 2%HIHS
Donor 1	26	22	40	45
Donor 2	31	33	21	20
Donor 3	35	25	50	50
	n=92	n=80	n=111	n=105
	25.5%	**22.2%**	**30.8%**	**29.2%**

Total Cloning Efficiency 26.9%

Figure 3. Cloning efficiency from healthy donor PBMCs using different culture methods.
Healthy donor's PBMCs were isolated via Ficoll and CD3+ CD8+ T cells were single cell sorted into 96 well plates containing irradiated feeder cells, 30ng/ml α-CD3, tissue culture supplement, and 50IU/ml IL-2. The media tested was IMDM 10%HS, IMDM 10%HIHS, IMDM 10%FBS 2%HS, and IMDM 10%HIFBS 2%HIHS. The number of cells sorted per culture condition was, A) 180 (60 cells/donor/condition) and, B) 360 (120 cells/donor/condition). Cloning efficiency was calculated from the number of clones transferred to a 24 well plate after two weeks of growing in 96 well plates divided by the total number of CD8+ T cells sorted for the specific media condition.

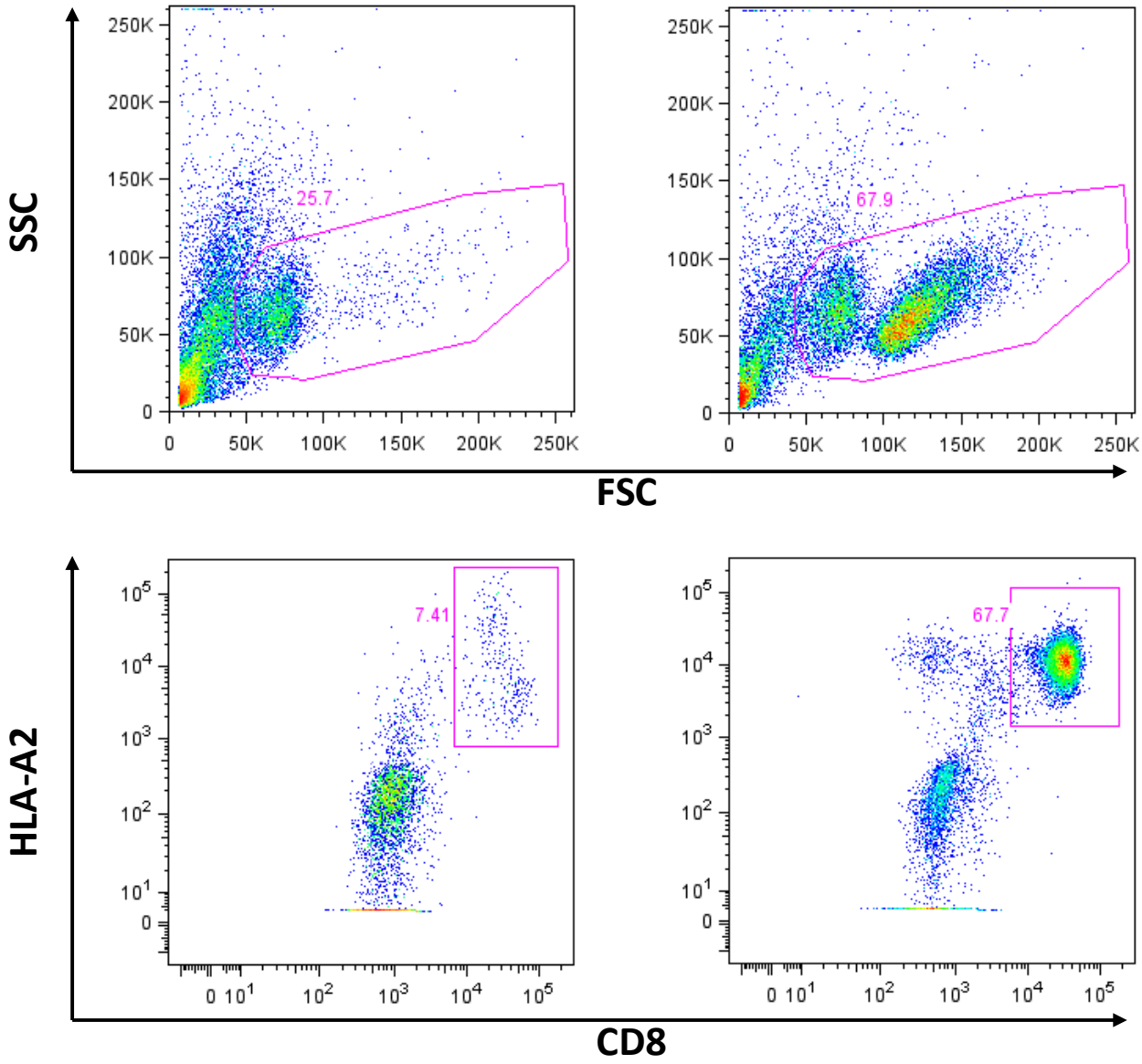

Figure 4. Flow plot demonstrating the differentiation of HLA-A2- feeder cells from HLA-A2+ breast cancer T cells.

Cells were labeled with CD8 and HLA-A2 antibodies for 20 minutes at room temperature and acquired in 96 well plates using a high through put (HTS) reader on a FACS Fortessa. The top plots show FSC versus SSC for a clone which is expanding at a slow rate (left) versus a robustly expanding clone (right) and the bottom plots demonstrate that we're able to distinguish CD8+ HLA-A2+ cells from patient donors versus HLA-A2-healthy donor cells.

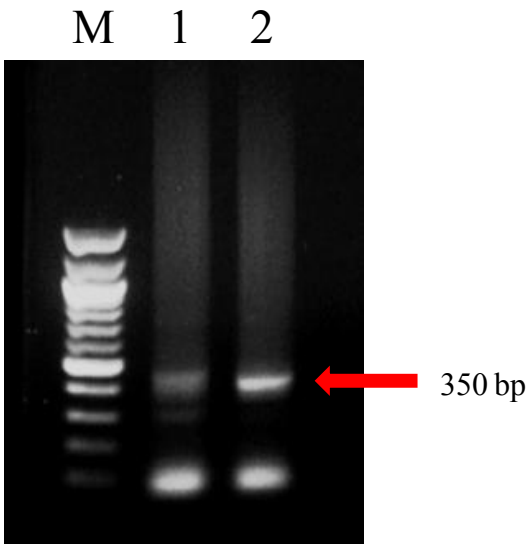

Figure 5. Gel electrophoresis of Patient BC81 with fused Vα and Vβ amplicons during a PCR reaction.
cDNA from BC81 clone #38 (Lane 1) and clone #49 (Lane 2) produced DNA fragments in length of 350bp as identified using a 100 bp ladder (M).

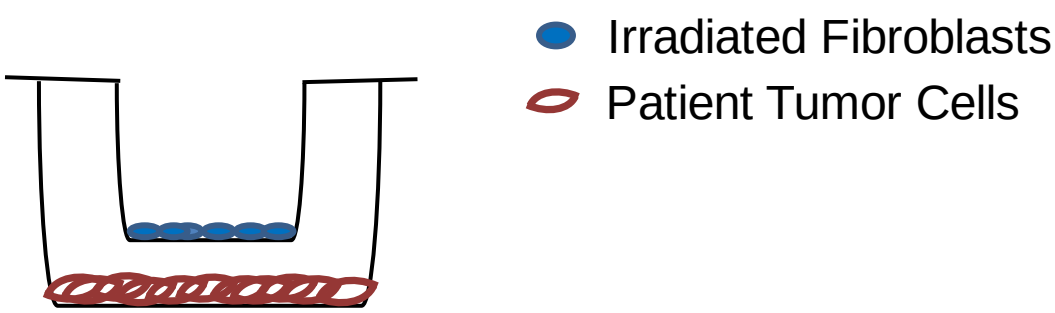

Figure 6. Illustration of a trans well culture system for patient tumor cells.
Patient tumor cells are seeded into the bottom of a plate. A transwell insert is placed on top of the well and irradiated fibroblast feeder cells are added into the transwell.

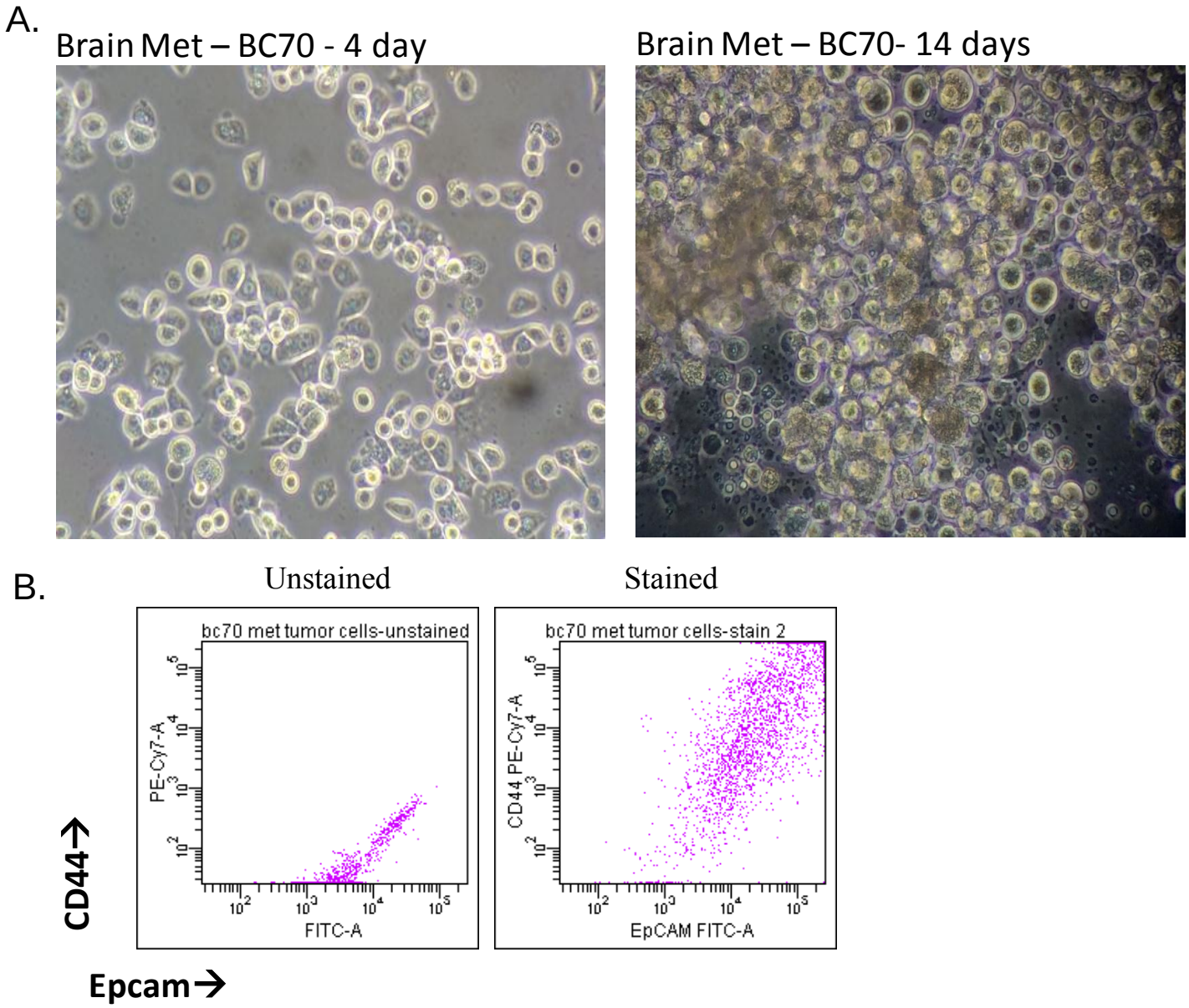

Figure 7. Brightfield image of breast cancer cell growth *in vitro*.
A). Image of cancer cells growing on a plate using a trans well culturing system. B) FACS plots demonstrating these cancer cells are EPCAM+ and CD44+.

PREVIOUSLY SHOWN

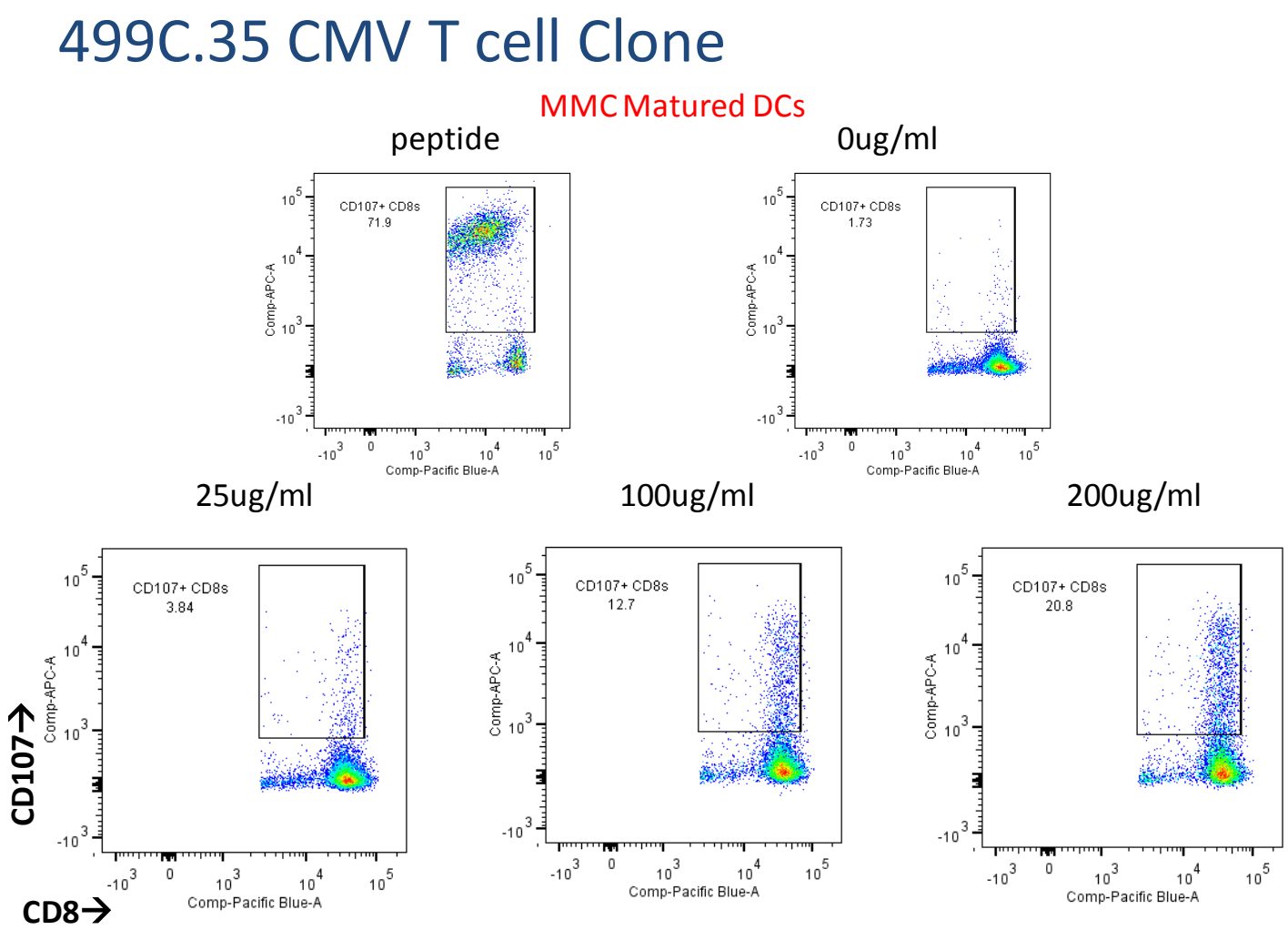

Figure 8. CMV specific T cell clone responses to CMV lysate.
A CMV specific T cell clone was stimulated with mDCs pulsed with increasing concentrations of CMV lysate (lower plots) or unpulsed mDCs (top right plot). Peptide pulsed mDCs were used as a positive control (top left). T cell reactivity was assessed by CD107 mobilization.

Cone 4
MMC

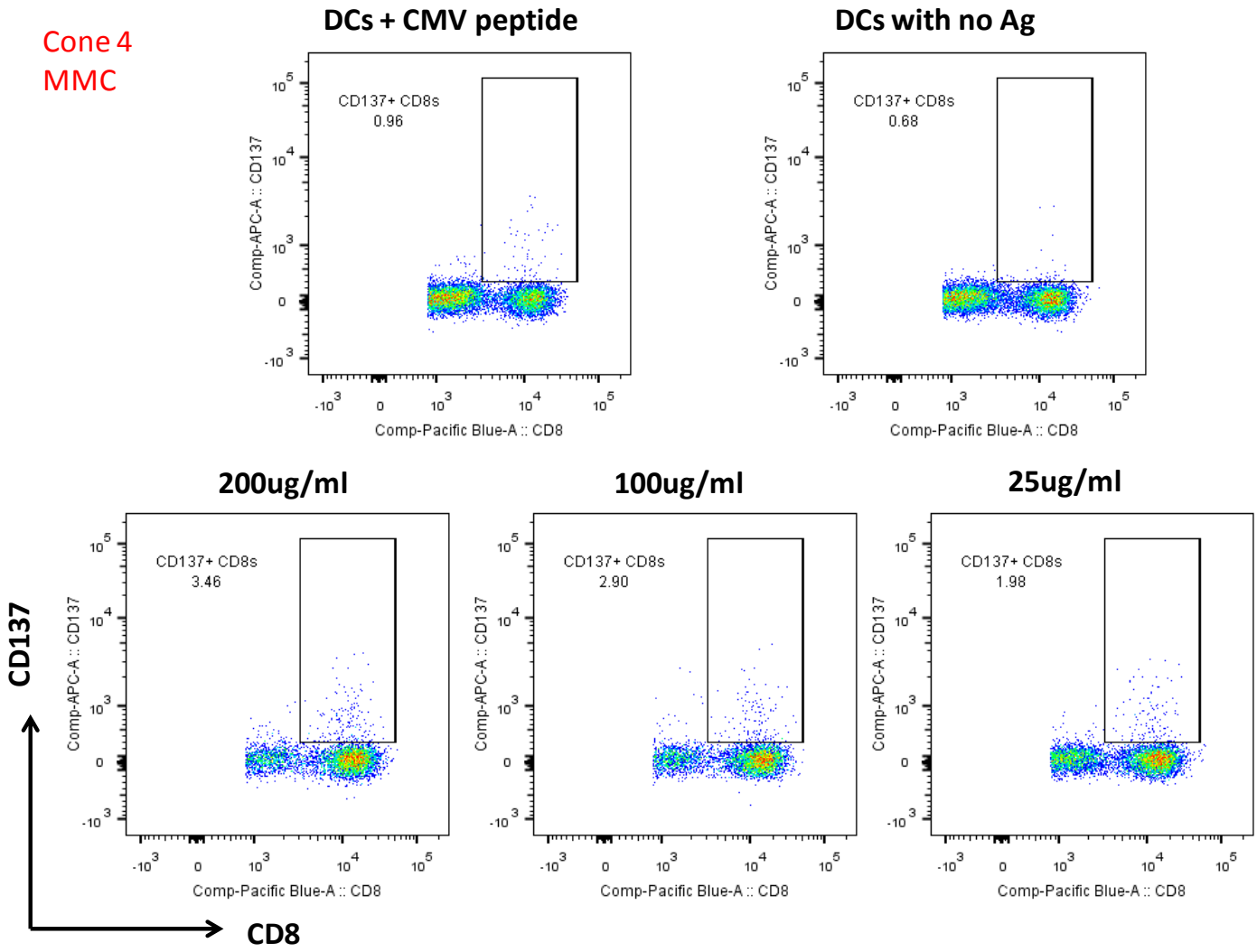

Figure 9. CMV specific CD8 T cells from a CMV reactive healthy donor to CMV lysate.
Autologous T cells were stimulated with mDCs pulsed with increasing concentrations of CMV lysate (lower plots) or unpulsed mDCs (top right plot). Peptide pulsed mDCs were used as a positive control (top left). T cell reactivity was assessed by CD137 expression after 24 hours.

Cone 4
MMC

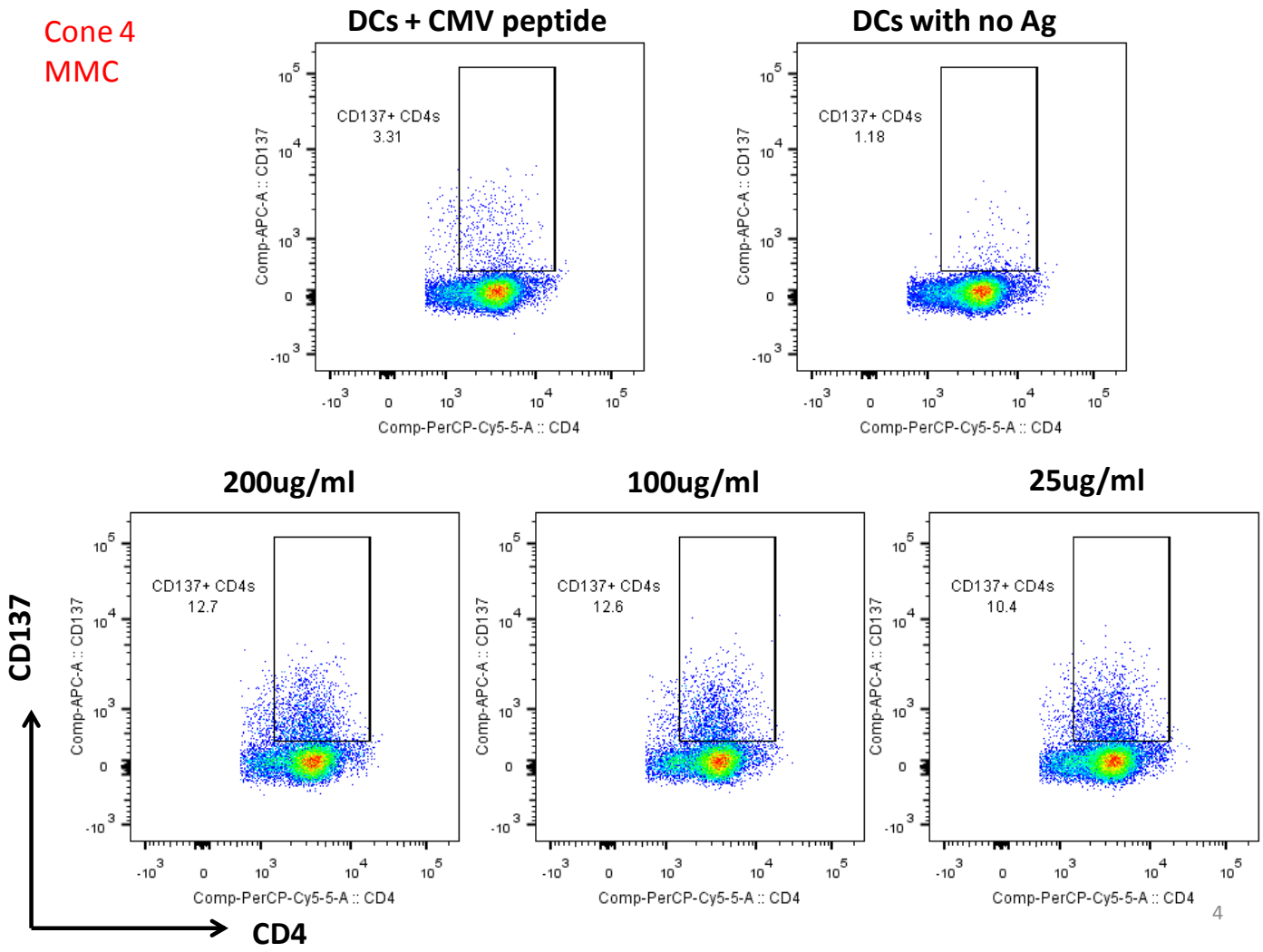

Figure 10. CMV specific CD4 T cells from a CMV reactive healthy donor to CMV lysate.
Autologous T cells were stimulated with mDCs pulsed with increasing concentrations of CMV lysate (lower plots) or unpulsed mDCs (top right plot). Peptide pulsed mDCs were used as a positive control (top left). T cell reactivity was assessed by CD137 expression after 24 hours.

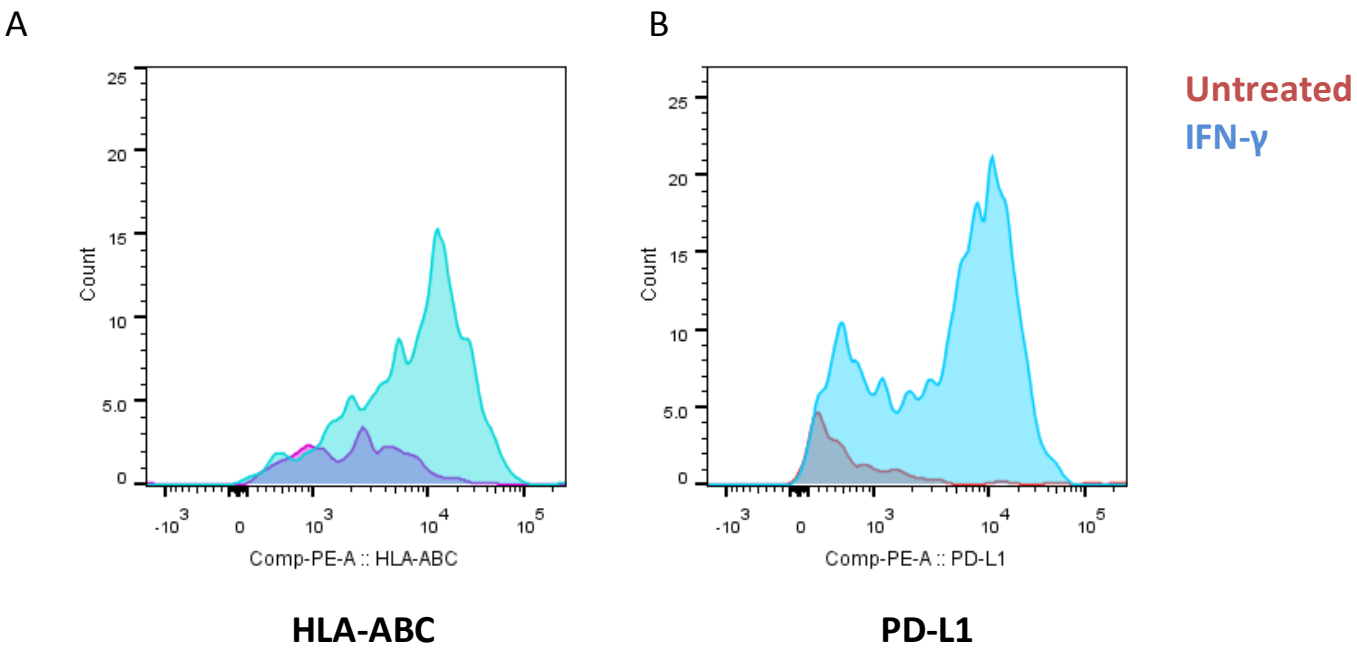

Figure 11. IFN-γ upregulates MHC I and PD-L1 expression on primary tumor cells.
Primary tumor cells remained untreated (red) or treated with 1000IU/ml of IFN-γ overnight. Expression levels of, A) HLA-ABC (MHC I) and, B) PD-l1 were assessed.

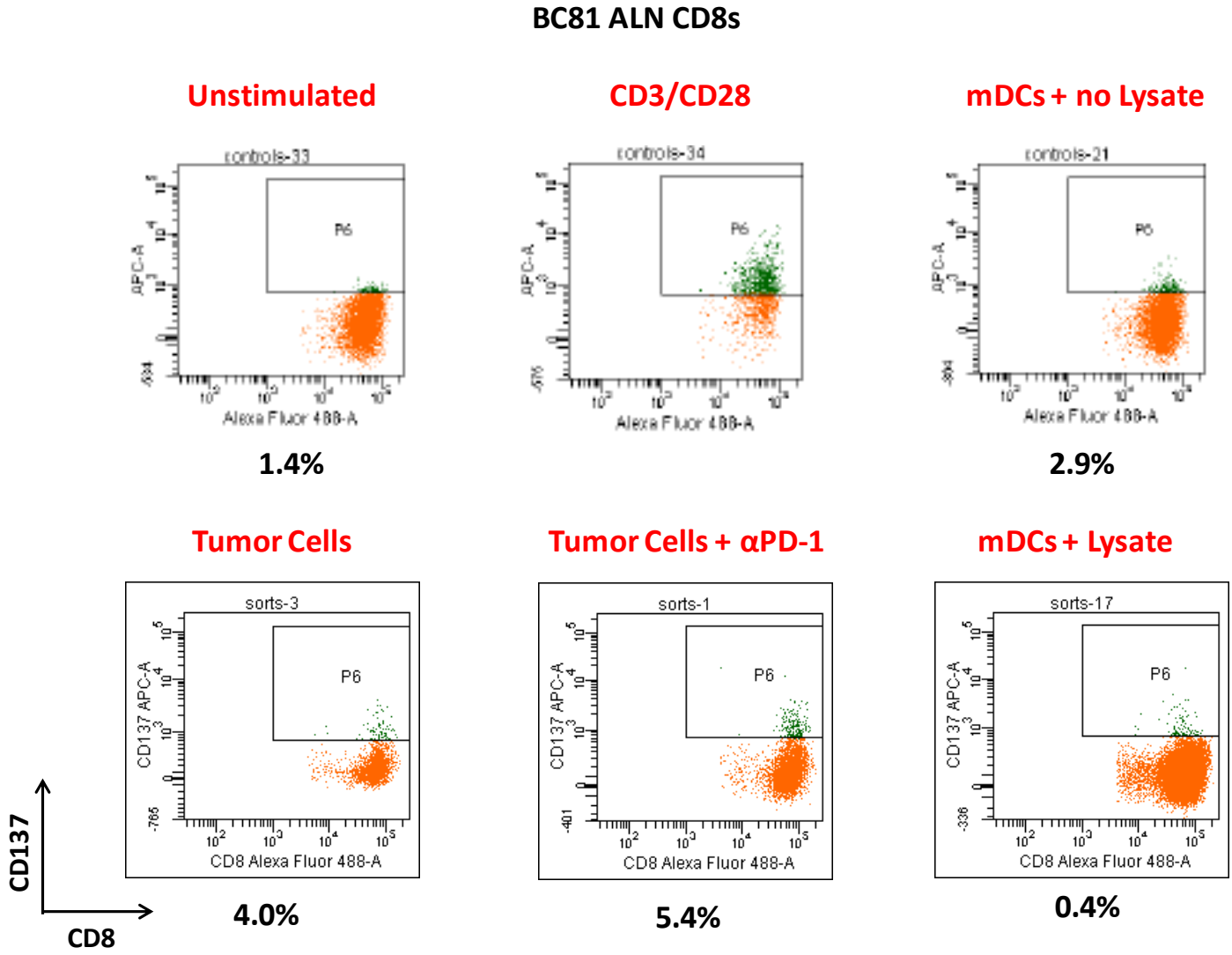

Figure 12. BC81 example of T cell reactivity to autologous tumor cells or breast cancer lysate pulsed mDCs.

T cells from patient BC81's axiallary lymph node (ALN) were co-cultured with autologous tumor cells with or without PD-1 blockade, or with mDCs pulsed with cancer cell line lysate (lower plots) over night and assessed for CD137 expression. Unstimulated T cells alone (upper left) and unpulsed mDCs (upper right) as well as T cells stimulated with α-CD3/CD28 beads served as negative and positive controls, respectively. The percentage ractive was calculated as CD137 expressing CD8 T cells minus CD137 expression in unstimulated controls of the same cells.

CD137+

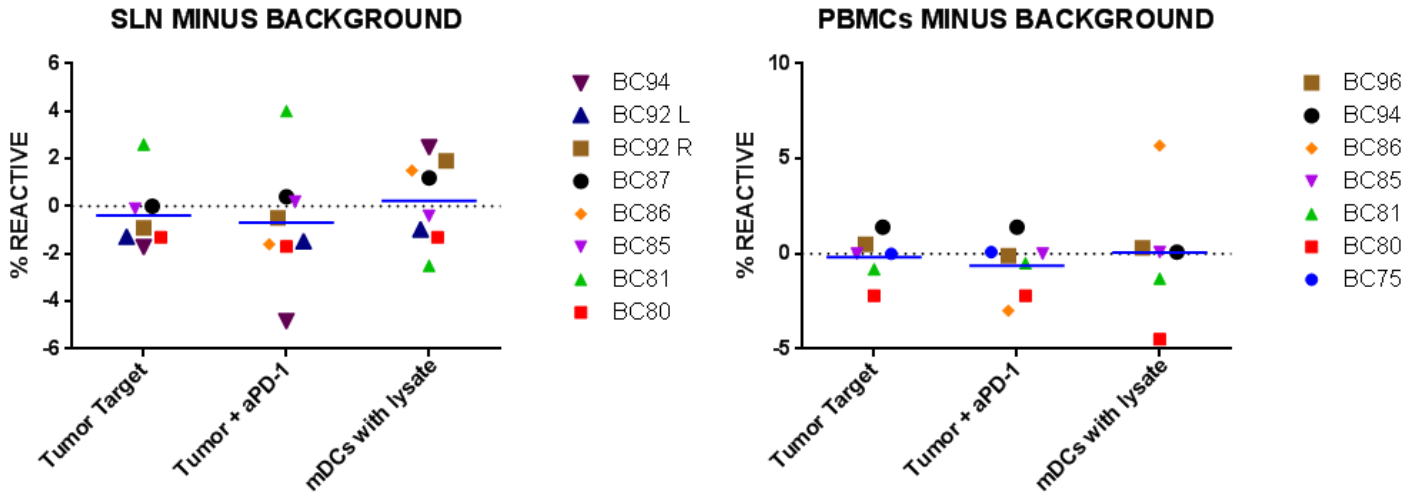

Figure 13. Summary of T cell reactivity to autologous tumor cells or breast cancer lysate pulsed mDCs in patients lymph nodes and PBMCs.

T cells from lymph node and/or PBMCs were co-cultured with autologous tumor cells with or without PD-1 blockade, or with mDCs pulsed with cancer cell line lysate over night and assessed for CD137 expression. The percentage of reactivity was calculated as CD137 expressing CD8 T cells minus CD137 expression in unstimulated controls of the same cell subset.